Metallothioneins (MTs) are a class of low molecular weight, cysteine-rich, metal-binding proteins ubiquitous in animals. They function in metal regulation and detoxification. An MT-like protein was separated by gel-permeation high-performance liquid chromatography (HPLC) in the least killifish Heterandria formosa exposed to 6 mg/L of Cd for 26 h. Western blot analysis showed that this protein cross-reacted with a cod–MT antibody. We quantified MT-like protein levels by the Cd–hemoglobin saturation assay in fish from two Cd-resistant and two control laboratory populations. Fish from the resistant populations had higher MT-like protein levels than those from the control populations; this difference was found for basal levels and for levels after 26 h of exposure to Cd. Our results suggest that the MT-like protein detoxified Cd in the least killifish and accounted for at least part of the increased resistance in the Cd-resistant laboratory populations.

Keywords—Cadmium-binding protein, Western blot, Cadmium saturation assay, Least killifish, Metal detoxification

INTRODUCTION

Metallothioneins (MTs) are a class of low molecular weight, metal-binding proteins widely distributed in a variety of organisms, including animals [1–3]. Metallothioneins are rich in cysteine and have few or no aromatic amino acids [4]; they are inducible by various factors including exposure to Cd, Zn, and Cu [2,5]. Several studies have shown that MT levels in metal-exposed organisms increase with increasing concentrations of metals [6,7], or with increased duration of exposure [8,9]. Metallothioneins were first reported in a fish species in 1974 [10]. Since then, MTs have been studied extensively in fish exposed to metals and other environmental stressors [11]. A role of MTs in the detoxification of metals was proposed when MT was initially described as a Cd-binding protein in the cortex tissue of horse kidney [12]. Since then, it has been shown that MTs function in the homeostatic regulation of essential metals such as Cu and Zn [13,14] and in the detoxification of nonessential metals and excess levels of essential ones [3,6].

Because MTs function in metal detoxification, differences among organisms in the rate of MT induction are likely to affect these organisms’ sensitivity to metal toxicity. Some studies have shown that the evolution of metal resistance might be tied to changes with respect to MTs [15,16]. Metal resistance could be associated with MT gene duplication [17] or amplification [15]. For example, Drosophila populations with MT gene duplications are more resistant to Cu and Cd [17]. Because the expression of MT genes is regulated at the transcriptional level [2], the MT gene duplications will result in elevated MT production following exposure to metals. Metal resistance has also been shown to be associated with an increase in MT mRNA [18] or elevated production of MT proteins [19]. Generally, the elevated production of MT decreases metal poisoning and thus translates into an increased resistance to specific metals.

Here we report on a metallothionein-like protein (MLP) and its role in Cd resistance in the least killifish Heterandria formosa. This freshwater fish is widely distributed in the southeastern United States [20]. In our ongoing research, this species has been subjected to laboratory selection for Cd resistance for multiple generations and shown a rapid response to selection [21]. By the sixth generation, the selection populations had a 200% greater resistance to Cd compared to the control populations. The goal of the current research was to determine whether changes in MT dynamics were responsible for this resistance difference.

MATERIALS AND METHODS

Test species

The base population of H. formosa was collected from Lake Martin (LA, USA) from October to December 1998. Three lines (S1-S3) were then subjected to laboratory selection for Cd resistance; each selection line was paired with a control line (C1-C3). Rather than having three replicate selection lines and three replicate control lines, pairing of lines precisely matched the number of individuals (and sex ratio) between a selection line and a control line, and because the Cd-exposure of fish from a pair of lines (control and selection) was done simultaneously and in the same (subdivided) aquarium [21]. All selection lines showed a rapid response to selection [21]. Fish were maintained on a 16:8-h light:dark cycle. Fish were fed Tetramin® flakes (Tetra Sales, Blacksburg, VA, USA) each morning and newly hatched brine shrimp (Inve Aquaculture, Grantsville, UT, USA) each afternoon.

Quantification and partial characterization of MLP was investigated in the F8 generation in two selection populations (S1 and S3) and two control ones (C1 and C3). At that point, the average resistance to Cd (quantified as time to 50% mortality when exposed to 6 mg/L of Cd) in the S1 and S3 lines was 81.3 h, while the average resistance in the C1 and C3 lines was 25.5 h.

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Cadmium exposure

Twenty to forty juvenile fish measuring 9 to 12 mm (standard length) were acclimated in reconstituted soft water (48 mg/L NaHCO₃, 30 mg/L CaSO₄, 30 mg/L MgSO₄, 2 mg/L KCl) [22] in a 38-L tank at room temperature with moderate aeration for about 24 h before the exposure. We used juvenile fish because this was the stage at which Cd-resistance was quantified and thus the stage most appropriate for studying the relationship between Cd-resistance and MLP dynamics. Fish were exposed to Cd at 6 mg/L (in the form of CdCl₂·2H₂O) for 26 h. The fish were then rinsed with reconstituted soft water and transferred to 10-ml scintillation vials and stored at −80°C until use. Metallothionein-like protein was analyzed in whole fish rather than specific tissues because we were interested in overall MTP levels and the size of the fish (with an average total wet wt of 0.2 g) made it unfeasible to work with individual organs and still have a reasonable number of replicates.

Purification of metallothionein-like protein

Fish stored at −80°C were allowed to thaw (while kept on ice). Several fish (about 1 g) were pooled and homogenized on ice using a glass/Teflon homogenizer and 2 ml homogenization buffer consisting of 20 mM Tris·HCl at pH 8.0, 200 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, and 5 mM dithiothreitol (DTT). The homogenates were centrifuged at 14,000 g for 30 min at 4°C (Centrifuge 5402, Eppendorf, Hamburg, Germany). The resulting supernatants were heat-denatured in boiling water for 10 min and centrifuged for 30 min. The supernatants from this step were subjected to acetone precipitation with pre-chilled acetone (final concentration: 80%) [23]. Each pellet was dissolved in 200 μl HPLC-buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, and 0.03% NaN₃) and stored at −20°C for later use.

The next step of the purification was conducted using gel-permeation high-performance liquid chromatography (GP-HPLC) with a TSK column (G3000SWXL; 7.8 mm × 30 cm; Tosoh Biosep, Montgomeryville, PA, USA). Twenty μl amounts of the heat-denatured and acetone-precipitated homogenates (see above) were processed by GP-HPLC at a flow rate of 0.5 ml/min with a run time of 35 to 40 min. Absorbance of eluant was monitored simultaneously and continuously at 254 and 280 nm by an ultraviolet detector (Waters 996 Photodiode Array detector, Waters, Milford, MA, USA). The chromatograms were recorded using Waters Millennium32 software. One-minute fractions were collected using a Gilson Fc 203B fraction collector (Gilson, Middleton, WI, USA). The Cd concentration of each fraction was determined by atomic absorption spectrophotometry (Perkin-Elmer model 1100B, Norwalk, CT, USA) equipped with graphite furnace (Model 700 HGA) and deuterium background correction. Each of the two fractions with the highest Cd levels (23±25 min retention time, Fig. 1) was further desalted and concentrated (to 203B fraction collector). Each of the two fractions with the highest Cd levels (23–25 min retention time, Fig. 1) was further desalted and concentrated (to 203B fraction collector). The above sample (in 20 μl of 20 mM Tris·HCl, pH 7.5) was mixed with 1.5 M Tris·HCl (pH 6.8 at 25°C), 20% SDS, 50% glycerol, and 0.2% bromophenol blue in order to obtain a 40-μl sample in 125 mM Tris, 1% SDS, 10% glycerol, and 0.01% bromophenol blue [25]. The buffer did not contain 2-mercaptoethanol because MT migrated on the gel as an irregular broad band in the presence of 2-mercaptoethanol [25]. The 40-μl sample was divided into half, and each 20-μl sample was applied to 15% SDS-PAGE gel and mobilized at a constant voltage of 78 V for 3 h in a buffer consisting of 25 mM Tris, 192 mM glycine, and 0.1% SDS. After electrophoresis, one gel was stained with Coomassie brilliant blue R-350 (ICN Biomedicals, Asse, Belgium). A second gel was incubated in 150 ml of 25 mM Tris pH 8.5, 192 mM glycine, 0.2% SDS and 5% 2-mercaptoethanol at 37°C for 1 h (without this treatment, transferred metallothionein is permeation separations of standard molecules) on molecular weight. The initial regression line was established using the following molecular-weight markers: Bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa). The regression line for these four standards had an R² value of 0.95. Rabbit MT was used to compare its characteristics to those of the MLP of H. formosa in GP-HPLC and SDS-PAGE.

SDS-PAGE and Western blot

The above sample (in 20 μl of 20 mM Tris·HCl, pH 7.5) was mixed with 1.5 M Tris·HCl (pH 6.8 at 25°C), 20% SDS, 50% glycerol, and 0.2% bromophenol blue in order to obtain a 40-μl sample in 125 mM Tris, 1% SDS, 10% glycerol, and 0.01% bromophenol blue [25]. The buffer did not contain 2-mercaptoethanol because MT migrated on the gel as an irregular broad band in the presence of 2-mercaptoethanol [25]. The 40-μl sample was divided into half, and each 20-μl sample was applied to 15% SDS-PAGE gel and mobilized at a constant voltage of 78 V for 3 h in a buffer consisting of 25 mM Tris, 192 mM glycine, and 0.1% SDS.

After electrophoresis, one gel was stained with Coomassie brilliant blue R-350 (ICN Biomedicals, Asse, Belgium). A second gel was incubated in 150 ml of 25 mM Tris pH 8.5, 192 mM glycine, 0.2% SDS and 5% 2-mercaptoethanol at 37°C for 1 h (without this treatment, transferred metallothionein is
not retained on a nitrocellulose membrane in the subsequent step [25]). The protein bands in the gel were electrophoretically transferred to the nitrocellulose membrane at a constant current of 90 mA for 3 h. The membrane was then incubated in blocking buffer (0.75% gelatin and 4% milk in phosphate buffer saline buffer consisting of 137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH2PO4, and 1.4 mM KH2PO4) for 30 min at room temperature. The membrane was then incubated with the first of two antibodies (1:500 dilution of rabbit anti-cod metallothionein; Cayman Chemical, Ann Arbor, MI, USA) for 2 h with gentle agitation at room temperature. The membrane was washed with 0.05% Tween-20 (Pierce Chemical, Rockford, IL, USA) in phosphate buffer saline 3 times for 10 min each before being incubated with the second antibody (1:2000 alkaline phosphatase conjugated goat anti-rabbit IgG) for 2 h with gentle agitation at room temperature. The membrane was washed three times. Phosphatase enzymatic activity was visualized using p-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoly1 phosphate in 100 mM Tris, 5 mM MgCl2, and 100 mM NaCl at pH 9.7.

Quantification of basal and induced MLP levels

The concentrations of MLP were determined using the Cd-hemoglobin affinity assay of Eaton and Cherrian [26] with the modification that stable Cd was used instead of 109Cd. To determine the basal level of MLP, 15 fish from each selection line and its paired control line were acclimated for 24 h in reconstituted soft water. They were then transferred individually to 10-ml scintillation vials that were frozen. The fish were transferred to clean water for 3 min, then individually stored in 10-ml scintillation vials that were frozen at −80°C.

Frozen fish were allowed to thaw while kept on ice. Individual fish were blotted dry, then weighed to the nearest 0.1 mg. Fish were homogenized in 10× (w/v) 20 mM Tris-HCl, pH 8.0 buffer with 200 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride and dithiothreitol 5 mM using a glass/Teflon homogenizer. The homogenates were transferred to 1.5-ml microfuge tubes and centrifuged at 10,000 g for 15 min at 4°C. The supernatants were heated denatured in boiling water for 2 min, immediately cooled on ice for 10 min, then centrifuged at 10,000 g for 3 min at 4°C. A 1-μl volume of a 100 μg/ml Cd stock solution was mixed with 99 μl of the cytosol from the previous step (in order to saturate the MLPs Cd-binding capacity), then incubated at room temperature for 20 min. After the incubation, 50 μl of a 2% bovine hemoglobin solution was added to the Cd-saturated sample (to bind excess Cd) and mixed. The hemoglobin was removed by heat-denaturing in boiling water for 2 min, cooling on ice, and centrifugation for 3 min at 10,000 g and 4°C. Bovine hemoglobin was added again to the sample, followed by 2 min of heating, 10 min on ice, and centrifugation at 14,000 g for 15 min at 4°C. The supernatant was transferred to a new microfuge tube. Meanwhile, four blanks (with homogenization buffer only), treated the same as the protein samples, were used to determine the efficiency of hemoglobin in removing excessive Cd.

The Cd concentration in the samples was analyzed by graphite furnace atomic absorption spectrophotometry. When quantifying MLP levels, we assumed that a fully saturated molecule of MLP binds seven atoms of Cd. Bovine hemoglobin efficiently removed all the unbound Cd from the solution, as indicated by a background absorbance on furnace atomic spectrophotometry.

Statistical analyses

All statistical analyses were performed using the Statistical Analysis System package (SAS®, Ver 8.01, Cary, NC, USA). Before analysis, data were checked for normality with the Shapiro-Wilk test and for lack of equality of variance using Levene’s test. There was no violation of assumptions of equal variance and normality. Analysis of variance (ANOVA) was used to test for differences in MLP levels between fish from the selection populations and those from the paired control populations, separately for the S1, C1 population pair and the S3, C3 pair. The analysis was conducted this way because each selection line was paired with its own control line (see above). Each ANOVA was a one-way ANOVA with population type (selected versus control) as the main effect and variation among fish within a population as the error term.

RESULTS

Characterization of metallothionein-like protein

When separating the heat-denatured extract of Cd-exposed fish by GP-HPLC, a large absorbance peak was observed at a retention time of 23.5 min, corresponding with elevated levels of Cd in fractions 22 to 26 (Fig. 1). Using the purification method with two acetone precipitation steps of the cytosol resulting from ultracentrifugation (100,000 g, 90 min at 4°C) [23], we also obtained a high-Cd-content fraction that eluted from the HPLC column at 24 min (results not shown). Under the same HPLC conditions, rabbit metallothionein eluted at 23.7 min (data not shown). The apparent molecular weight of the MLP extracted from H. formosa, estimated from the regression of molecular weight on retention time, was about 23 kDa. The protein peak at 23.5 min had a large ratio (~4) of absorbance at 254 nm to absorbance at 280 nm (A254/A280 ratio).

Analysis by SDS-PAGE showed that the high-Cd-content fraction contained two high-molecular weight bands (~50 and 70 kDa), a band with an apparent molecular weight of 17 kDa, and some lower molecular-weight material (<14.2 kDa; Fig. 2). We also observed that the rabbit MT migrated a distance similar to that of the 17 kDa band on the SDS gel (results not shown). Western blot analysis showed that the high-Cd-content fraction had only one protein band (which corresponded to the 17 kDa protein on the SDS-PAGE gel) that cross-reacted with the polyclonal rabbit anti-cod metallothionein antibody (Fig. 2).

Basal and induced levels of MLP

In the two sets of paired lines investigated in the F8 generation (S1, C1, S3, and C3), the basal levels of MT-like proteins in the selection lines were higher than those in the paired control lines (Fig. 3; C1 vs S1, F1,28 = 5.31, p = 0.029; C3 vs S3, F1,28 = 10.57, p = 0.002). Basal levels of MLP in the fish from the selection lines S1 and S3 were 40.3 and 46.1% higher than those in the paired control lines (C1 and C3, respectively). Fish from both the selection lines and the control lines responded to Cd exposure with an increase in MLP level. After 26 h of exposure to Cd at 6 mg/L, MT-like protein levels in the two selection lines were higher than those in their paired control populations (C1 vs S1, F1,28 = 5.98, p = 0.021; C3 vs S3, F1,28 = 4.31, p = 0.047). The levels of MLP after 26 h of
exposure were 49.5 and 32.2% greater than those in the control lines. In both the control and selection lines, MLP levels following the 26 h of exposure were 4-fold higher than levels before exposure.

**DISCUSSION**

*Metallothionein-like protein in H. formosa*

Our results showed that a low molecular mass (molecular wt of ~23 kDa), heat stable, Cd-binding protein was obtained from the cytosol of the least killifish exposed to Cd at a concentration of 6 mg/L for 26 h. This MT-like protein eluted in the gel-permeation step at almost the same retention time as rabbit MT, indicating a close similarity of molecular weight between the MLP and rabbit MT. Furthermore, the apparent molecular weight of the MLP (~23 kDa) is similar to the 20 to 23 kDa estimates for Cd-binding proteins of a scallop (*Argopecten irradians*) [27] and common mussel (*Mytilus edulis*) [28,29] also obtained by GPC. Metallothioneins generally have a molecular weight of about 6 to 7 kDa, but the 23-kDa protein may represent a polymer. Dimers of MT have been inferred from GPC results in several other studies [30,31]. The fact that the 23 kDa estimate obtained by GPC is higher than the 17 kDa estimate obtained by SDS-PAGE and Western blot may be a consequence of the rod shape of metallothioneins (causing a metallothionein to migrate in gel permeation as would be expected from a larger-sized spherical protein). The ultraviolet absorption spectra of the corresponding peak in our GP-HPLC separation were characterized by high ratios of absorbance (254 nm to 280 nm), which suggests the existence of metal-thiolate bonds [31]. Low molecular weight, metal-binding proteins similar to MT have been found in many taxa, including fish [3]. The high-Cd–content fractions obtained by GP-HPLC contained a low molecular weight protein (apparent molecular wt ~17 kDa on 15% SDS-PAGE gel), two high molecular weight bands, and a significant amount of low molecular weight material (<14.2 kDa). Similar SDS-PAGE results, with both low and high molecular weight proteins present in a Cd-binding fraction obtained by gel permeation chromatography and anion-exchange HPLC, have been reported for other Cd-exposed fish [32]. In our study, only the 17 kDa protein cross-reacted with the polyclonal anti-cod MT antibody. This antibody has been shown to cross-react with MTs from other fish species (albeit with varying affinity) [33]. The band observed in the Western blot analysis indicated that there was a weak reactivity of the MLP from the least killifish with the rabbit anti-cod MT antibody. Again, this 17 kDa protein could be a dimer, because dimerization is common in SDS-PAGE electrophoresis [34]. MT-like proteins with similar molecular weights estimated from SDS-PAGE electrophoresis also have been reported for the channel catfish (*Ictalurus punctatus*) [32] and the zebra mussel (*Dreissena polymorpha*) [35].

**Metallothionein-like proteins and cadmium resistance**

Metallothionein-like protein concentrations in fish of both control and selection populations increased about 4-fold after exposure to Cd for 26 h. Numerous reports have shown that MTs are induced by exposure to Cd [3 and references therein]. The magnitude of the increase in MLP in this study was similar to values reported for other studies [7,9]. Both basal and induced MTs are important for the detoxification of Cd [2]. In our study, both basal and induced MT levels were higher in fish from the selection populations than in those from their paired control populations. Thus, fish from the selection populations had a higher capacity for Cd detoxification (which would be expected to bring about a higher Cd resistance) compared to fish of the control populations. Similar results were obtained in a Cd resistant strain of *Saccharomyces cerevisiae*, which exhibited both higher rates of basal *CUP1* (a MT gene in yeast) transcription and Cd-induced *CUP1* expression [18].

When quantifying Cd accumulation in least killifish, we found that a Cd accumulation of 45 μg/g dry weight was lethal to these fish [36]. If our MLP is assumed to be fully saturated with Cd only (with 1 MLP molecule binding 7 atoms of Cd), the induced MLP could bind up to 26.5% (in fish from the selection populations) or 18.9% (in fish from the control populations) of the lethal level of Cd. The first value is similar to the one reported for *Mytilus edulis* exposed to Cd [9]. Although the selection line fish’s capacity to bind more of the accumulated Cd to a MLP may account for all their increased Cd-resistance, the maximum measured Cd-binding capacity (26.5%) implies that much of the accumulated Cd was not

![Fig. 2. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and Western blot analysis of the combination of the two highest-cadmium-content fractions from gel permeation liquid chromatography. Lane A: Western blot analysis; arrow shows the location of the band cross-reacting to the anti-cod metallothionein. Lane B: the concentrated cadmium-rich fractions on 15% SDS-PAGE gel. Lane C: standard molecular weight proteins on 15% SDS-PAGE gel.](image)

![Fig. 3. Basal metallothionein-like protein (MLP) levels and MLP levels after exposure to 6 mg/L of cadmium for 26 h in fish from two selections lines (S1 and S3) and their paired control lines (C1 and C3). * p < 0.05; ** p < 0.01. Comparisons were made within paired lines (C1 vs S1; C3 vs S3).](image)
Role of metallothionein-like protein in cadmium resistance

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